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Three-dimensional protein assembly directed by orthogonal non-covalent interactions

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In this report, orthogonal non-covalent interaction strategy based on specific recognition between sugar and protein, and host-guest interaction, was employed to construct artificial three dimensional (3D) protein assemblies in laboratory.

Protein self-assemblies inspire board research interest, due to their crucial role in nature, as well as their potential applications as biocompatible and biodegradable materials. Biologists demonstrated that self-assembled proteins structures, e.g. filament, microtubule, virus capsid, etc. are essential to various functions, including material and energy transportation, gene encapsulation etc. Meanwhile, these biomaterials have precise and uniform nanostructure, which renders them significant advantages over other organic structures made from polymers or small molecules.

In the past decades, creative strategies have been developed to construct artificial protein nanostructure, including controlling protein symmetry, developing fusion protein or genetically modifying protein surface with functional groups. Different kinds of non-covalent interactions i.e. metal coordination, host-guest interaction and hydrophobic interactions, have been used to obtain protein nanostructures, including nanowires, nano-rings, helical tubes and three-dimensional structures etc. For example, Liu and co-workers modified dimeric glutathione S-transferase with tripeptide Phe-Gly-Gly (FGG) on its symmetrical sides and then constructed protein nanowire structure by connecting FGG with Cucurbit[8]uril (CB[8]).

To our knowledge, in nature proteins often form 1D, 2D or 3D nanostructures via multiple interactions orthogonally, i.e. the different interactions work independently. Biodiversity contributes to the smart world significantly by its own way. Thus it is desirable for chemists to achieve orthogonal self-assembly of proteins in laboratory for biomimetic study. In the past decades, various supramolecular structures starting mostly from different synthetic building blocks, have been constructed via orthogonal self-assembly. However, as far as we know, few reports demonstrated the formation of regular protein assemblies in nm or µm scale via orthogonal interactions. A beautiful example came from Ward and Hayashi, in which apomyoglobin and streptavidin were linked alternatively via dual molecular recognitions. Recently we demonstrated that by using a synthetic small molecular ligand composed of both sugar and rhodamine B (RhB), proteins self-assembled into 3D crystal with interpenetrating networks or helical microtubes, driven by protein-sugar interaction and π−π stacking of RhB. It was found that in this self-assembly process, the protein-sugar interaction took place first followed by the π−π stacking. To make the two interactions happen independently, i.e. to achieve orthogonal protein self-assembly, a new ligand is designed in the current research, in which π−π stacking of RhB was replaced by the molecular recognition based on cucurbit[8]uril (CB[8]). Then a new kind of artificial protein assembly was...
constructed via orthogonal non-covalent interactions, i.e. the recognition between sugar-protein and the host-guest interaction based on CB[8].

To perform orthogonal self-assembly of proteins, Concanavalin A (ConA), a homotetrameric protein exhibiting D2 symmetry, was selected as a model (concentration calculated as its monomer in this work). On each of its monomer, there is a sugar-binding site, favoring α-mannopyranoside (Man), which is one of the orthogonal interactions. For the other one, we chose the inclusion complexation based on CB[8]. Due to its high binding ability to guest molecules, many kinds of supramolecular polymers have been constructed. Thus ligands containing Man and guest species to CB[8] were designed. As shown in Fig. 1, ligands NapMan and AzoMan having naphthyl or azobenzene groups as guests respectively, were synthesized. As reported in literature, one CB[8] could bind two naphthalene molecules at the same time with binding constant approximately $10^{11}$-$10^{12}$ M$^{-2}$ or with one azobenzene and one dipyridyl salt (DDPS) forming 1:1:1 supramolecular complex. The two pairs of interactions were quite successful in making different kinds of supramolecular systems.

In order to determine the ConA/Man recognition and the host-guest interactions based on CB[8] quantitatively and particularly to explore whether one interaction can be realized without the interference of the other, series of isothermal titration calorimetry (ITC) measurements were performed as follows. First, the binding constant of NapMan with ConA was measured as $1.15 \times 10^7$ M$^{-1}$ (Fig. 2a), while fitting the ITC result of NapMan with CB[8] with a two-site binding model gave high binding constants of $K_1 = 1.09 \times 10^6$ M$^{-1}$, $K_2 = 8.18 \times 10^5$ M$^{-1}$ and 2:1 stoichiometry of NapMan and CB[8] (Fig. S1). The interaction was also supported by $^1$H NMR (Fig. S2a) and UV-vis spectroscopy (Fig. S2b, c), and the latter further proved the 2:1 binding ratio of NapMan and CB[8]. Then the binding constants were measured again in the ternary system ConA/NapMan/CB[8]. As shown in Fig. 2b, the equivalent mixture of ConA and NapMan (0.6 mM) in aqueous solution, in which Man/ConA binding already existed, was titrated to the solution of CB[8] (0.02 mM). A two-site binding model gave binding constants of $K_1 = 4.56 \times 10^6$ M$^{-1}$, $K_2 = 3.51 \times 10^5$ M$^{-1}$, which were close to the constants of NapMan and CB[8] without ConA. Meanwhile, mixture of CB[8] (0.5 mM) and NapMan (1 mM), was titrated to solution of Con A (0.05 mM) with the binding constant measured as $1.4 \times 10^6$ M$^{-1}$ (Fig. S3), which was also consistent to that of NapMan and ConA without CB[8]. Moreover, the results from single mode ITC (SIM-ITC) proved that the interactions took place simultaneously. As shown in Figure 2c, the heat quantity (3.65 µcal) of NapMan titrating to the mixture of ConA/CB[8] almost equals to the sum of the heat released of equivalent NapMan titrating to ConA (0.93 µcal) and to CB[8] (2.79 µcal) separately. Similarly, when AzoMan was used as ligand, the consistency of binding constants in the four component system ConA/AzoMan/CB[8]/DDPS to the corresponding ones in literature also supports the possibility of orthogonal self-assembly (Fig. S4-S5).

To perform the orthogonal protein self-assembly, an equimolar NapMan and ConA was mixed with 0.5 eq. CB[8] in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (20 mM) ([NaCl] = 20 mM, [CaCl$_2$] = 5 mM, [MnCl$_2$] = 5 mM), and the final concentration of NapMan and ConA was 0.15 mM. After the three components were mixed together, assembled structure formed with its size increasing along time until precipitates formed after 3 h, as monitored by dynamic light scattering (DLS).
(Fig. 2d). Similar results for the self-assembly of the four component system ConA/AzoMan/CB[8]/DDPS were also obtained from DLS (Fig. S6). After the assembly was formed, addition of 10 eq mannose or 10 eq adamantane hydrochloride (Ada) to the ConA/AzoMan/CB[8]/DDPS solution, induced disassociation of the assembly because of supramolecular competition (Fig. S7). Moreover, the size distribution of the assembly can be controlled by addition of free mannose. When 5 equiv. mannose was added while ConA/AzoMan/CB[8]/DDPS were mixed together, the assembly size kept stable around several hundred nano-meter without precipitation within at least 12 h. However, when 10 equiv. mannose was used in the same experiment, no assembly was observed within 12 h (Fig. S8).

Fig.2 ITC results of raw and integrated data for titration of (a) NapMan (1.5mM) to Con A (0.1mM) (calculated as monomer) and (b) mixture of ConA and NapMan (0.6 mM) to CB[8] (0.02 mM) in aqueous solution at 20°C. (c) SIM-ITC data of 0.59 mM NapMan titrating into 0.05 mM ConA, 0.025 mM CB[8], and the mixture of 0.05 mM ConA and 0.025 mM CB[8]. DLS results of ConA/NapMan solution after addition of CB[8] at different time interval.

Fig.3 (a) HR-TEM image of ConA/AzoMan/DDPS/CB[8] (diffraction pattern inserted). (b) TEM and (c) HR-TEM images of ConA/NapMan/CB[8] assembly. The fast Fourier transform result was inserted in c. (d) The zoomed-in image in the white box in c. e) Possible mechanism of the protein assembly formation.

More importantly, TEM was employed to observe the morphology of the assembly with negative staining (2% uranyl acetate). As shown in Fig.3, the images clearly showed that the assemblies of both ConA/AzoMan/CB[8]/DDPS (Fig. 3a) and ConA/NapMan/CB[8] (Fig. 3b) were stacked layers. On the edge of the former structure (Fig. 3a), more than five layers were clearly observed. Each layer has a rather regular shape, although different layers could not stack into regular macroscopic objects. Diffraction pattern (inset of Fig. 3a) showed their crystalline state of protein assembly. Similarly, diffraction pattern of NapMan/CB[8]/ConA system was also clearly observed (inset of Fig. 3c). Furthermore, under high resolution TEM (Fig. 3c, d), aligned regular patterns inside the layers of proteins were clearly observed. The size of the repeating unit was about 6.4 nm in ConA/NapMan/CB[8] assembly. According to all above results, a possible
mechanism of orthogonal protein self-assembly was shown in Fig. 3e, i.e. ConA interacted with NapMan via the specific ConA/Man interaction, while CB[8] induced the dimerization of NapMan via CB[8]/Nap interaction. These components packed regularly into the protein layers, which finally stacked into macroscopic precipitates.

Finally, light responsiveness of ConA/AzoMan/DDPS/CB[8] assembly was explored. It is known that under UV light, trans-azobenzene isomerizes into its cis-isomer and loses its binding ability to CB[8], while under visible light the cis-isomer can be transformed back to the trans one.\(^\text{17}\) We found that this feature could persist in the protein assembly containing AzoMan. As shown in Fig. 5B, after UV irradiation (\(\lambda = 365\) nm) for about 30 min, the absorption intensity of ConA/AzoMan/DDPS/CB[8] at about 345 nm almost disappeared, while the absorption around 428 nm became higher (red line in Fig. S8), showing the successful conversion of AzoMan from trans- to cis-isomer. Then the sample was placed under visible light and UV-vis spectroscopy was used to trace the isomerism of AzoMan from cis to trans in the assembly. It was found that within 230 min, the absorption at 345 nm was steady recovered to the absorption intensity before UV irradiation (black line in Fig. S9), while that at 428 nm went down to almost no absorption. The UV-vis absorption spectra clearly indicated that the isomerism of azobenzene had been reserved in the assembly. However, we did not find obvious disappearance of the assembly under UV irradiation, which means that dissociation of the protein assembly did not occur completely. This phenomenon was unexpected. We speculate that the regular pattern inside the assembly might be too tight to be disrupted by the isomerism of azobenzene.

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**Notes and references**


